CLAIMS

What is claimed is:

1. A method for detecting an oxidation enzyme comprising the steps of:

providing an organic substrate and an oxygen donor;

supplying a test enzyme comprising a protein-containing material selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

introducing the test enzyme to the organic substrate and the oxygen donor;

furnishing a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

testing for the detectable composition, wherein the presence of the detectable composition indicates that the test enzyme is an oxidation enzyme.

2. A method of claim 1, wherein the test enzyme is introduced to the organic substrate and the oxygen donor under reaction conditions which are selected to evaluate the ability of the test enzyme to mediate the addition of oxygen to the substrate.

3. A method of claim 2, wherein the reaction conditions are varied.

4. A method of claim 2, wherein the coupling enzyme is furnished under coupling conditions selected to promote formation of a polymeric oxygenated composition comprising two or more of the same oxygen-containing products joined to each other.

5. A method of claim 1, wherein the organic substrate is an aromatic compound.

6. A method of claim 1, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

7. A method of claim 1, wherein the oxygen donor is molecular oxygen.

8. A method of claim 1, wherein the oxygen donor is a peroxide.

9. A method of claim 8, wherein the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide.

10. A method of claim 1, wherein the coupling enzyme is used to bring together molecules of the oxygen-containing product to form a polymeric composition that is detectable using at least one of ultraviolet light, a color change, fluorescence and luminescence.

11. A method of claim 1, wherein the coupling enzyme is a peroxidase enzyme.

12. A method of claim 6, wherein the coupling enzyme is a peroxidase enzyme.

13. A method of claim 8, wherein the coupling enzyme is a peroxidase enzyme.

14. A method of claim 1, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

15. A method of claim 1, wherein the coupling enzyme is a laccase enzyme.

16. A method of claim 1, wherein the organic substrate is an aromatic compound, the oxygen donor is a peroxide and the coupling enzyme is a peroxidase enzyme.

17. A method of claim 16, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

18. A method of claim 16, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, an peroxidase Novozyme® 502.

19. A method of claim 1, further comprising the step of providing a chemiluminescent agent.

20. A method for detecting an oxygenase enzyme comprising the steps of:

providing an aromatic organic substrate and a peroxide oxygen donor;

supplying a test enzyme comprising a protein-containing material selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

introducing the test enzyme to the organic substrate and the oxygen donor;

furnishing a peroxide coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

testing for the detectable composition by evaluating at least one of ultraviolet light, a color change, fluorescence, and luminescence,

wherein the presence of the detectable composition indicates that the test enzyme is an oxygenase enzyme.

21. A method of claim 20, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

22. A method of claim 20, wherein the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide.

23. A method of claim 20, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

24. A method of claim 20, further comprising the step of providing a chemiluminescent agent.

25. A method of claim 23, further comprising the step of providing a chemiluminescent agent.

26. A method of claim 20, wherein the chemiluminescent agent is luminol.

27. A method of claim 23, wherein the chemiluminescent agent is luminol.

28. A method of claim 20, wherein the oxygenase enzyme is one of a dioxygenase enzyme and a monooxygenase enzyme.

29. A method of claim 20, wherein the reaction catalyzed by the oxygenase enzyme is one of a hydroxylation reaction, an epoxidation reaction, and a sulfoxidation reaction.

30. A method of claim 1, wherein the oxygenase enzyme is selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

31. A method of claim 20, wherein the oxygenase enzyme is selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

32. A method of claim 20, wherein the organic substrate is naphthalene.

33. A method of claim 20, wherein the oxygen donor is hydrogen peroxide.

34. A method of claim 20, wherein the coupling enzyme is horseradish peroxidase.

35. A method of claim 1, wherein the introducing step comprises introducing the organic substrate and oxygen donor to host cells that express at least one of a test enzyme and a coupling enzyme.

36. A method of claim 20, wherein the introducing step comprises introducing the organic substrate and oxygen donor to host cells that express at least one of a test enzyme and a coupling enzyme.

37. A method of claim 1, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

38. A method of claim 20, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

39. A method of claim 35, wherein the host cells are bacterial cells.

40. A method of claim 36, wherein the host cells are bacterial cells.

41. A method of claim 39, wherein the host cells are *E. coli* cells.

42. A method of claim 40, wherein the host cells are *E. coli* cells.

43. A method of claim 45, wherein the host cells are yeast cells.

44. A method of claim 36, wherein the host cells are yeast cells.

45. A method of claim 43, wherein the host cells are *S. cerevisiae* cells.

46. A method of claim 44, wherein the host cells are *S. cerevisiae* cells.

47. A method of claim 20, wherein:

the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin;

the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide; and

the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

48. A method of claim 47, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

49. A method of claim 48, further comprising the step of providing a chemiluminescent agent.

50. A method of claim 1, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the absence of at least one coenzyme or ancillary protein.

51. A method of claim 20, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the absence of at least one of its coenzymes or ancillary proteins.

52. A method of claim 47, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the absence of at least one of its coenzymes or ancillary proteins.

53. A method of claim 50, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH).

54. A method of claim 50, wherein at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

55. A method of claim 51, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH).

56. A method of claim 51, wherein at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

57. A method of claim 1, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the presence of one or more cofactors.

58. A method for detecting an oxygenase enzyme comprising the steps of:

providing an aromatic organic substrate and a peroxide oxygen donor;

supplying a test enzyme comprising a protein-containing material selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

introducing the test enzyme to the organic substrate and the oxygen donor in the absence of at least one of its coenzymes or ancillary proteins;

furnishing a peroxide coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

testing for the detectable composition by evaluating at least one of ultraviolet light, a color change, fluorescence, and luminescence,

wherein the presence of the detectable composition indicates that the test enzyme is an oxygenase enzyme.

59. A method of claim 63, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH).

60. A method of claim 58, wherein at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

61. A method of claim 58, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the presence of one or more cofactors.

62. A method of claim 61, wherein at least one cofactor is selected from the group consisting of thiamine (vitamin B1), ferrous chloride ($FeCl_2$) and delta-aminolevulinic acid (ALA).

63. A method of claim 58, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase,

toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

64. A method of claim 38, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH), or at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

65. A method for evaluating a test enzyme for its ability to mediate production of an oxygenated product when introduced to an organic substrate in the presence of an oxygen donor, comprising the steps of:

providing a host cell which is capable of expressing a test enzyme;

furnishing a vector which encodes the test enzyme;

inserting the vector into the host cell to provide a transformed host cell that expresses the test enzyme;

supplying an oxygen donor;

introducing an organic substrate to provide a reaction with the oxygen donor in the presence of the test enzyme to produce an oxygenated product;

reacting the oxygenated product in the presence of a coupling enzyme to form a polymeric oxygenated product; and

detecting the polymeric oxygenated product by testing for at least one of fluorescence, luminescence, ultraviolet light, and a color change.

66. A method of claim 65 wherein the host cell further expresses the coupling enzyme.

67. A method of claim 65 comprising the further step of introducing a second vector which expresses the coupling enzyme into the host cell.

68. A method of claim 65 comprising the further step of reacting the polymeric oxygenated product with a chemiluminescent agent to produce a chemiluminescent compound, and

the detecting step comprises evaluating the luminescence of the chemiluminescent compound.

69. A method of claim 65, wherein the test enzyme is a mutant oxygenase enzyme.

70. A method of claim 69, wherein the mutant oxygenase enzyme is mutant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase enzymes.

71. A method of claim 65, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

72. A method of claim 65, wherein the oxygen donor is a peroxide.

73. A method of claim 65, wherein the coupling enzyme is a peroxidase.

74. A method of claim 69, wherein the mutant oxygenase enzyme is a mutant of a P450 enzyme.

75. A method of claim 74, wherein the oxygen donor is a peroxide.

76. A method of claim 74, wherein the coupling enzyme is a peroxidase.

77. A method of claim 74, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin, the oxygen donor is a peroxide, and the coupling enzyme is a peroxidase.

78. A method of claim 75, wherein the coupling enzyme is horseradish peroxidase.

79. A method of claim 74, wherein the P450 enzyme is a $P450_{cam}$ enzyme.

80. A method of claim 1, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

81. A method of claim 20, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

82. A method of claim 58, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

83. A method of claim 65, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

84. A method of claim 65, wherein the vector comprises a variant of the $P450_{cam}$ nucleotide sequence of FIG. 3A **[SEQ. ID. NO. 1]**.

85. A method of claim 65, wherein the vector encodes a mutation of the $P450_{cam}$ amino acid sequence of FIG. 3B **[SEQ. ID. NO. 2]**.

86. A method of claim 66, wherein the second vector comprises the horseradish peroxidase nucleotide sequence of FIG. 23 **[SEQ. ID. NO. 15]**.

87. A method of claim 66, wherein the second vector encodes the horseradish peroxidase amino acid sequence of FIG. 23 **[SEQ. ID. NO. 16]**.

88. A method of screening for oxygenase enzymes comprising the steps of:

providing host cells;

furnishing a plurality of vectors each of which encodes a test enzyme;

inserting each vector into one or more host cells to provide corresponding transformed cells that each express a corresponding test enzyme;

introducing each test enzyme to an oxygen donor and an organic substrate under conditions which provide for a reaction between the substrate and donor to produce an oxygenated product;

reacting the oxygenated product in the presence of a coupling enzyme to form a polymeric oxygenated product; and

detecting the polymeric oxygenated product by testing for the presence or degree of at least one indicator selected from the group consisting of fluorescence, luminescence, ultraviolet light, and a color change, wherein detection of the polymeric oxygenated product indicates that a corresponding test enzyme is an oxygenase.

89. A method of claim 88, wherein a plurality of the oxygenase enzymes are compared to each other by evaluating the corresponding degrees of detected indicator.

90. A method of claim 88, further comprising the step of reacting the polymeric oxygenated product with a chemiluminescent agent to form a chemiluminescent composition, and wherein the detecting step comprises testing for luminescence of the chemiluminescent composition.

91. A method of claim 88, wherein each vector encodes a test enzyme that is a variant of an oxygenase enzyme.

92. A method of claim 88, wherein each test enzyme is a variant or an oxygenase enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

93. A method of claim 88, wherein each vector encodes a test enzyme that is a variant enzyme obtained by at least one of random mutagenesis, specific mutagenesis, directed evolution, DNA shuffling, and error-prone polymerase chain reaction.

94. A method of claim 88, wherein the oxygen donor is a peroxide.

95. A method of claim 92, wherein the oxygen donor is a peroxide, the coupling enzyme is a peroxidase, and the organic substrate is a aromatic compound selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

96. A method of claim 58, wherein the detecting step includes image analysis of detected ultraviolet light, color change, fluorescence or luminescence.

97. A method of claim 65, wherein the detecting step includes image analysis of detected ultraviolet light, color change, fluorescence or luminescence.

98. A method of claim 88, wherein the detecting step includes image analysis of detected ultraviolet light, color change, fluorescence or luminescence.

99. A method of claim 88 wherein one or more steps are automated.

100. A method of claim 88, wherein the steps of the method are performed independently for each test enzyme and corresponding transformed cells.

101. A method of claim 100, wherein one or more steps of the method are preformed contemporaneously for each test enzyme and corresponding transformed cells.

102. A method of claim 88, wherein the steps of the method are repeated until at least one oxygenase enzyme is identified.

103. A method of claim 102, wherein each test enzyme is a variant of at least one identified oxygenase enzyme.

104. A method of claim 88, wherein each transformed cell expresses the coupling enzyme.

105. A method of claim 88, wherein the introducing step comprises providing the transformed cells with a supply of organic substrate and oxygen donor.

106. A method of claim 105, wherein the introducing step comprises providing the transformed cells with a supply of organic substrate and oxygen donor.

107. An oxygenase enzyme variant obtained by the method of claim 1.

108. An oxygenase enzyme variant obtained by the method of claim 20.

109. An oxygenase enzyme variant obtained by the method of claim 47.

110. An oxygenase enzyme variant obtained by the method of claim 58.

111. An oxygenase enzyme variant obtained by the method of claim 65.

112. An oxygenase enzyme variant obtained by the method of claim 88.

113. An oxygenase enzyme variant obtained by the method of claim 103.

114. An oxygenase enzyme variant obtained by the method of claim 104.

115. A P450 oxygenase enzyme variant having at least one mutation at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme.

116. A P450 oxygenase enzyme variant of claim 115, in which glutamic acid is changed to lysine.

117. A P450 oxygenase enzyme variant having at lease one mutation at a position corresponding to position 280 of the amino acid sequence of a wild-type P450 enzyme.

118. A P450 oxygenase enzyme variant of claim 117, in which arginine is changed to lysine.

119. A P450 oxygenase enzyme variant having at least one mutation at a position corresponding to position 242 of the amino acid sequence of a wild-type P450 enzyme.

120. A P450 oxygenase enzyme variant of claim 119, in which cysteine is changed to phenylalanine.

121. A P450 oxygenase enzyme variant having at least one mutation at a position corresponding to any of positions 242, 280 and 331 of the amino acid sequence of a wild-type P450 enzyme.

122. A P40 oxygenase enzyme variant having at least one mutation in which:

glutamic acid is changed to lysine at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme;

arginine is changed to leucine at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme; and

cysteine is changed to phenylalanine at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme.

123. A sequence-conservative variant of an enzyme of claim 122.

124. A function-conservative variant of an enzyme of claim 122.

125. An oxygenase enzyme variant encoded by a first polynucleotide that hybridizes to a second polypeptide encoded by an enzyme of claim 122 under high stringency conditions.

126. A method for evolving an oxidation enzyme comprising the steps of:

supplying an organic substrate and an oxygen donor;

providing at least one host cell that expressed a DNA sequence that encodes a provided oxidation enzyme capable of promoting the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

generating a test enzyme library comprising a plurality of oxidation enzyme mutants, each of which is a variant of at least one provided DNA sequence;

expressing a plurality of variants in host cells to produce a plurality of test enzymes from the library;

introducing each test enzyme to the organic substrate and the oxygen donor;

furnishing a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product;

testing for the detectable composition, wherein the presence of the detectable composition identifies the test enzyme as an oxidation enzyme;

selecting at least one identified oxidation enzyme by comparison with at least one provided oxidation enzyme according to ~~at least~~ one property.

127. A method of claim 126, wherein the method is repeated, using at least one identified oxidation enzyme as a provided oxidation enzyme.

128. A method of claim 126, wherein the generating step includes the use of error-prone PCR to provide mutants.

129. A method according to claim 126, wherein the oxygen donor is hydrogen peroxide, and the selecting step includes a comparison of enzyme activity.

130. An P450 enzyme evolved according to claim 129, and having an improved enzyme activity or stability in comparison with a provided P450 enzyme.

131. A P450 enzyme for use with hydrogen peroxide and obtained by a method of claim 126.

132. A method for evaluating the reaction conditions for an oxidation catalyst comprising the steps of:

providing an oxidation catalyst;

supplying an organic substrate and an oxygen donor;

introducing the catalyst to the organic substrate and the oxygen donor under each of a plurality of reaction conditions, to form a plurality of test combinations;

furnishing each test combination with a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product;

examining the test combinations for production of the detectable composition, wherein the reaction conditions are evaluated according to differences in at least one of the relative rates and amounts of production of the detectable composition.

133. An evolved P450 enzyme variant having a catalytic activity at least twice as active as a corresponding P450 wild-type enzyme in facilitating the oxidation of a substrate in the presence of hydrogen peroxide.

134. An evolved P450 enzyme variant having a catalytic activity at least ten times as active as a corresponding P450 wild-type enzyme in facilitating the oxidation of a substrate in the presence of hydrogen peroxide.

135. An evolved P450 enzyme variant having a catalytic activity at least twice as stable as a corresponding P450 wild-type enzyme.

136. An evolved P450 enzyme variant having a catalytic activity at least ten times as stable as a corresponding P450 wild-type enzyme.

137. A method for detecting an oxidation catalyst comprising the steps of:

providing an organic substrate and an oxygen donor;

supplying a test catalyst selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

introducing the test catalyst to the organic substrate and the oxygen donor;

furnishing a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

testing for the detectable composition, wherein the presence of the detectable composition indicates that the test catalyst is an oxidation catalyst.

138. A method of claim 137, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

139. A method of claim 1, wherein the oxygen donor is a peroxide.

140. A method of claim 137, wherein the coupling enzyme is used to bring together molecules of the oxygen-containing product to form a polymeric composition that is detectable using at least one of ultraviolet light, a color change, fluorescence and luminescence.

141. A method of claim 137, wherein the coupling enzyme is at least one of a peroxidase enzyme and a laccase enzyme.

142. A method of claim 137, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

143. A method of claim 137, wherein the organic substrate is an aromatic compound, the oxygen donor is a peroxide and the coupling enzyme is a peroxidase enzyme.

144. A method of claim 137, further comprising the step of providing a chemiluminescent agent.

145. A method of claim 137, wherein a plurality of indicated oxidation catalysts are compared to each other by evaluating the corresponding testing for detectable composition.

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